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Cloning Human Pyrroline-5-carboxylate Reductase cDNA by Complementation in *Saccharomyces cerevisiae**

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Pyrroline-5-carboxylate reductase (EC 1.5.1.2) catalyzes the NAD(P)H-dependent conversion of pyrroline-5-carboxylate to proline. We cloned a human pyrroline-5-carboxylate reductase cDNA by complementation of proline auxotrophy in a *Saccharomyces cerevisiae* mutant strain, DT1100. Using a HepG2 cDNA library in a yeast expression vector, we screened 10^6 transformants, two of which gained proline prototrophy. The plasmids in both contained similar 1.8-kilobase inserts, which when reintroduced into strain DT1100, conferred proline prototrophy. The pyrroline-5-carboxylate reductase activity in these prototrophs was 1–3% that of wild type yeast, in contrast to the activity in strain DT1100 which was undetectable. The 1810-base pair pyrroline-5-carboxylate reductase cDNA hybridizes to a 1.85-kilobase mRNA in samples from human cell lines and predicts a 319-amino acid, 33.4-kDa protein. The derived amino acid sequence is 32% identical with that of *S. cerevisiae*. By genomic DNA hybridization analysis, the human reductase appears to be encoded by a single copy gene which maps to chromosome 17.

Pyrroline-5-carboxylate (P5C)[1] reductase (EC 1.5.1.2) catalyzes the reduction of P5C to proline in an NAD(P)H-dependent reaction which is both the first committed and final step in proline synthesis. P5C reductase activity is present in the cytosol of virtually all mammalian tissues and cultured cells. In addition to its role in proline synthesis, P5C reductase, together with the other enzymes of P5C and proline metabolism, may influence the ratios of oxidized/reduced pyridine nucleotides (1).

Two lines of evidence suggest that there may be two (or more) forms of P5C reductase. First, kinetic studies show that P5C reductase from various tissues displays different cofactor preferences. P5C reductase from human erythrocytes (2), bovine retina (3), and rat lens (4) has a 20–60-fold lower K_m for NADPH *versus* NADH and has a 5–12-fold higher affinity for P5C with NADPH as cofactor (2). Purified erythrocyte P5C reductase utilizes NADPH exclusively when both pyridine nucleotides are present at physiologic concentrations (2). By contrast, P5C reductase from cultured human fibroblasts (5) and a lymphoblastoid cell line (LHN cells) (6) has a similar affinity for either cofactor and an affinity for P5C which is not dependent on the choice of cofactor. Second, the sensitivity of P5C reductase to inhibitors is tissue-specific. The enzyme from cultured human fibroblasts and LHN cells is inhibited by proline but not by $NADP^+$; the converse is true for P5C reductase from erythrocytes, lens, and retina (2). Based on these differences in kinetic characteristics and sensitivity to inhibitors, Phang and his colleagues (1, 2, 5–7) suggested that there are different forms of P5C reductase in various tissues. Furthermore, they propose that the reductase reaction serves different metabolic roles in these tissues (for review, see Ref. 1). In fibroblasts, cartilage, and other tissues with a high requirement for proline, the primary function of the reaction is to synthesize this amino acid (7). Alternatively, in erythrocytes, where the enzyme is inhibited by $NADP^+$ and there is no requirement for proline synthesis, the primary function of the reaction may be to produce $NADP^+$ necessary for activity of the hexose monophosphate shunt.

As an initial step in determining the molecular basis for the tissue-specific characteristics of P5C reductase and the factors which influence its functional roles, we set out to clone a human P5C reductase cDNA and ultimately the structural gene(s). At the time of institution of these studies, primary sequence information for P5C reductase was available only from microorganisms (8–10)[2] and soybean (11). Small amounts of P5C reductase had been purified from mammalian sources, but the quantity was insufficient for antibody production or sequence determination (2–4). Therefore, we elected to utilize a functional cloning strategy, namely complementation in a *Saccharomyces cerevisiae* mutant strain lacking the reductase. We reasoned that P5C reductase should be amenable to this cloning strategy. The human protein appears to be a homopolymer comprised of moderately sized (~30 kDa) subunits (2). Mutant strains of *S. cerevisiae* lacking P5C reductase activity have been well characterized (12) and are auxotrophic for proline, providing a selection system for complementation. Appropriate human cDNA libraries in a yeast expression vector recently have been described (13).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M77836.

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[1] The abbreviations used are: P5C, pyrroline 5-carboxylate; bp, base pair(s); LHN, lymphoblastoid, SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; HEK, human embryonic kidney; kb, kilobase(s).

[2] M. C. Brandriss and D. A. Falvey, submitted for publication.

Using similar complementation strategies, the human homolog for the cell cycle control gene (*cdc2*) (14), a CCAAT-binding transcription factor (15), and three multifunctional genes involved in *de novo* purine synthesis (13, 16) have been cloned.

In this report, we describe the isolation and characterization of human P5C reductase cDNA by complementation in *S. cerevisiae* and mapping of the human structural gene.

MATERIALS AND METHODS AND RESULTS[3]

DISCUSSION

By complementation of an *S. cerevisiae* P5C reductase mutant strain, we have cloned two independent cDNAs encoding human P5C reductase. Phenotypic complementation occurred despite the relatively low level of P5C reductase activity measured in the transformants (Table II). Either wild type *S. cerevisiae* have far more P5C reductase activity than required for growth on a medium lacking proline or the enzymatic activity measured *in vitro* does not accurately reflect that *in vivo*. Clearly, the low P5C reductase activity in the prototrophic transformant (KD100) does not result from a low level of P5C reductase mRNA (Fig. 5*A*). This lack of correlation between the levels of mRNA and enzymatic activity could be explained by inefficient translation of the message, lack of necessary post-translational processing, instability of the human protein in yeast, or a negative interaction of the truncated yeast P5C reductase subunits with the wild type human subunits. Antibodies against the human P5C reductase protein will be useful in distinguishing among these possibilities.

Our longest human P5C reductase cDNA is 1.8 kb in length and has 11 bp upstream of the first AUG. We propose that this first AUG marks the start of translation based on two lines of evidence. First, the context provides a reasonable Kozak consensus sequence (36). There is an A at position −3, a G at −6, and purine at +4 (Fig. 3). Second, use of the next downstream AUG would eliminate amino-terminal protein sequence which has homology with the reductases from other species (Fig. 4). Initiation of translation at the first AUG gives a 957-bp open reading frame encoding a protein of 319 amino acids with a predicted molecular mass of 33.4 kDa. This is consistent with the size of the P5C reductase monomer purified from human erythrocytes which was estimated to be ~30 kDa by SDS-polyacrylamide gel electrophoresis (2).

The predicted amino acid sequence for human P5C reductase has considerably homology to the reductases from microorganisms and soybean (Fig. 4). There are blocks of 7–14 amino acids in which the identity is >75%. Presumably, these regions are important for the structure and/or function of the enzyme. We were unable to identify with certainty an NADPH binding site that fits the consensus based on several reductase enzymes (I_ _G_GTGIAPF_ _F[~100 amino acids] Y_CG_ _ _ _M) (37). Perhaps this reflects the fact that P5C reductase can use either NADPH or NADH. Also, it is interesting that the human protein extends approximately 40 carboxyl terminal amino acids beyond any regions of identity with the reductase sequences from microorganisms and soybean.

P5C reductase mRNA is present in a wide variety of cell lines, including HepG2 cells, skin fibroblasts, HEK cells, HeLa cells, AD12 cells, and MCF 7 cells, in agreement with the presence of P5C reductase activity in virtually all mammalian cells and tissues (38). The high level of enzyme activity in HepG2 cells as compared to fibroblasts can be partially accounted for by the greater amount of reductase mRNA in the former. The poly(A$^+$) RNA from HepG2 cells exhibits at

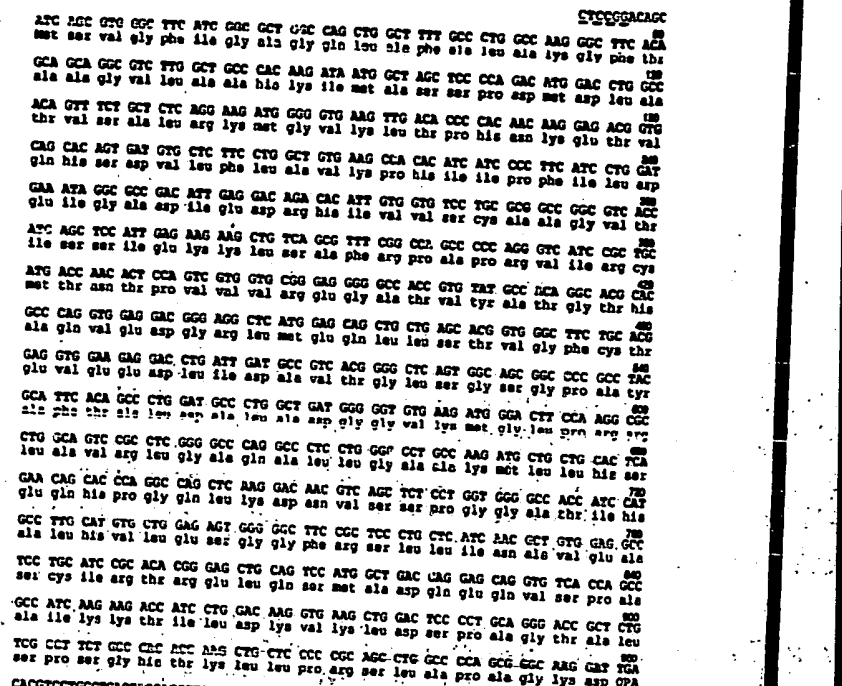

FIG. 3. **The nucleotide sequence of human P5C reductase cDNA.** The bases indicated by the *dashed underline* were determined from the sequence of huP5CR.2. The predicted amino acid sequence is shown below. The proposed polyadenylation signal is *underlined*. The nucleotide sequence is numbered from the A of the first ATG.

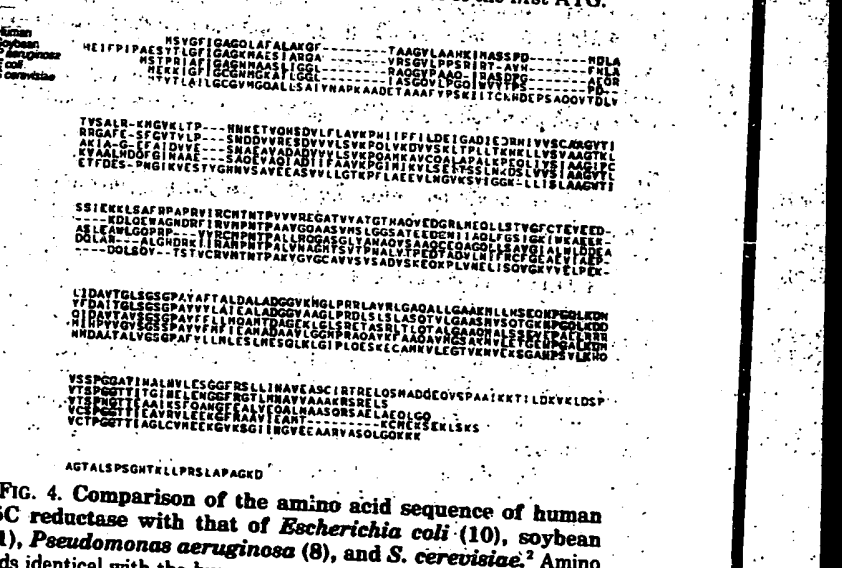

FIG. 4. **Comparison of the amino acid sequence of human P5C reductase with that of *Escherichia coli* (10), soybean (11), *Pseudomonas aeruginosa* (8), and *S. cerevisiae*.**[2] Amino acids identical with the human sequence are *shaded*. Alignment was done using the Needleman and Wunsch alignment algorithm program.

[3] Portions of this paper (including "Materials and Methods", "Results," Tables I and II, and Figs. 1, 2, 5, and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

least two bands when probed with huP5CR.1 cDNA. These different species could be due to differential splicing of the P5C reductase mRNA which, if the pattern varied in a tissue-specific fashion, could explain the different enzymatic forms of the protein. Alternatively, the two P5C reductase transcripts could reflect the use of two sites for polyadenylation. In this regard, the proposed polyadenylation signal in both phuP5CR.1 and phuP5CR.2, AUUAAA, is not the canonical AAUAAA (39).

We examined the human genomic organization of the human P5C reductase gene by Southern blot analysis. The gene appears to be single copy with a relatively simple structure, as evidenced by the low number of hybridizing fragments even at low stringency (Fig. 6). If the tissue-specific differences in P5C reductase kinetics and inhibitor sensitivity were due to multiple P5C reductase genes, we would expect a more complex pattern of genomic fragments.

A human genetic disease caused by an abnormality of P5C reductase has not yet been recognized. The expected phenotypic features for deficiency of P5C reductase might include runting, chondrodysplasia (7), cataracts (1), impaired lactation (40), and/or hemolytic anemia (41). Results with two human/rodent hybrid cell mapping panels showed that the reductase gene mapped to human chromosome 17. Examination of the phenotypes of the human genetic diseases mapped to this chromosome (42) or to its major murine counterpart (mouse chromosome 11) (43) does not reveal obvious candidates.

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SUPPLEMENTAL MATERIAL TO: Cloning Human Pyrroline-5-Carboxylate Reductase cDNA by Complementation in *Saccharomyces cerevisiae*

Kristiann M. Dougherty, Marjorie C. Brandriss and David Valle

MATERIALS AND METHODS

Yeast Strains and Culture

The *S cerevisiae* strains used in this study are described in TABLE I. Strain DT1100 is isogenic with strain MB1433 except for deletion of that portion of the *pro3* gene which follows codon 83 (codons 84-286). For transformation, the DT1100 strain was grown in minimal medium [0.2 g/100 ml $(NH_4)_2SO_4$, 0.15 g/100 ml yeast nitrogen base without amino acids and $(NH_4)_2SO_4$ (Difco), 2 g/100 ml glucose] supplemented with proline (8.7 mM) and uracil (0.2 mM). Transformants were selected on minimal plates (minimal medium plus 2 g/100 ml Difco agar) supplemented with 8.7 mM proline, replica plated to and scored on minimal plates without proline. Growth rates in minimal medium (supplemented with uracil [0.2 mM] and tryptophan [0.8 mM] for MB1433) were measured in log phase culture.

TABLE I: *S CEREVISIAE* STRAINS USED IN THIS STUDY

Strain	Genotype	Plasmid	Source
MB1433	*MATα trp1 ura3-52*	none	Brandriss [17]
DT1100	*MATα trp1 ura3-52 pro3::TRP1*	none	Brandriss [12]
KD100	*MATα trp1 ura3-52 pro3::TRP1*	pPRO+.1	this work

cDNA Library

We obtained a human cDNA library, constructed from size-selected (>500 bp) HepG2 (a human hepatoma cell line) cDNA in a yeast expression vector obtained from A. Brake [13]. The promotor and terminator are provided by the *S cerevisiae* glyceraldehyde-3-phosphate dehydrogenase gene. This 2 μM-derived vector contains origins of replication and selectable markers (*URA3* and amp^R^), appropriate for propagation and maintenance in either *S cerevisiae* or *E coli*.

Transformation of Yeast and *E coli*

The entire HepG2 library was used to transform strain DT1100 as described by Schiestl and Gietz [18], with an incubation at 42° C for one hour. Competent *E coli* 294 cells were prepared and transformed as described [19].

Preparation of Plasmid and Insert DNA and Sequencing

DNA was isolated from transformed yeast as described [20] and used to transform 294 cells as above. Plasmid DNA was prepared from *E coli* by standard methods [21]. After digesting the plasmids with Bgl II, the inserts were isolated in low melting point agarose (BRL) and ligated into the BamHI site of pGEM4 (Promega Biotec). Sequencing was performed with the T7 polymerase kit (Pharmacia) or the Sequenase kit (US Biochem) according to the manufacturers' instructions.

Nucleic Acid Analysis

Human genomic DNA was prepared from peripheral lymphocytes as described [22]. Total cellular RNA was isolated from tissue culture cells by guanidium thiocyanate extraction [23]. Poly A+ RNA was isolated by oligo d(T)-cellulose chromatography [24]. RNA was prepared from *S cerevisiae* as by Chapman [25].

DNA transfer and hybridization were as described by Mitchell et al [26]. Reduced stringency hybridizations were performed in a solution of 35% (v/v) formamide, 1 M NaCl, 10% (w/v) dextran sulfate and 1% (w/v) sodium dodecyl sulfate (SDS). Washes, at reduced stringency, were done twice in 2 x SSC (1 x SSC - 150 mM NaCl, 15 mM sodium citrate), 1% SDS for 10 min at room temperature, twice in 2 x SSC, 1% SDS at 50° C for 30 min and twice in 2 x SSC at room temperature for 30 min. RNA transfer and hybridization were performed using GeneScreen Plus membranes (NEN) following manufacturer's protocols. The probes for these blots were prepared by radiolabelling the appropriate DNA fragments isolated in low melting point agarose using the random hexamer procedure [27]. The huP5CR.1 cDNA probe is the 1.8 kb Bgl II insert of pPRO+.1. A 1.6 kb M85 mouse tubulin cDNA [28] and a 1.1 kb Bam HI / Hind III fragment of the *S cerevisiae* actin gene [29] were used to control for quality of RNA on the Northern blots. Autoradiograms were quantitated by densitometry using an LKB Ultrascan XL laser densitometer.

Chromosomal Localization

Two human/rodent hybrid cell line mapping panels were used to determine the chromosomal location of the P5C reductase gene. The first, generously provided by T. Mohandas [30], was screened by Southern blotting of Hind III digested DNA and the human P5C reductase cDNA as probe. The second (BIOS Corporation) was screened with the polymerase chain reaction (PCR) using Taq polymerase (Cetus) and primers corresponding to nucleotides 714 - 732 and the complement of nucleotides 1048 - 1066 of the P5C reductase cDNA (FIGURE 3).

Cell Culture

Human cell lines were grown in Eagles' minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum. The cell lines used in this study include skin fibroblasts, human embryonic kidney cells (HEK) (CRL1573), HepG2 cells, MCF7 cells (breast carcinoma cell line), AD12 cells (retinoblasts) (gift of RTMJ Vaessen [31]) and HeLa S3 cells (CCL2.2).

Cell Homogenates and P5C Reductase Assay

Saturated overnight cultures of MB1433, DT1100 or KD100, grown in minimal medium with appropriate supplements, were diluted to an OD_{600} of 0.3 and harvested at an OD_{600} of 0.6. Pellets were resuspended in 0.1 M potassium phosphate buffer, pH 6.8, 1 mM phenylmethylsulfonyl fluoride and the cells were disrupted by vortexing for 30 seconds with glass beads x 4 at 4° C. The beads were washed with additional 0.1 M potassium phosphate buffer pH 6.8 to yield a final homogenate volume of 1 ml. The homogenates were dialyzed overnight in 2 liters of 0.1 M potassium phosphate buffer pH 6.8 at 4° C.

Homogenates of cultured human cells were prepared as described [32]. Protein concentration was determined with the bicinchoninic acid reagent (Pierce) using bovine serum albumin as the standard. The P5C reductase activity was assayed radioisotopically as described [33] except the final concentration of NADH was 2.1 mM.

Reagents

All chemicals not otherwise specified were obtained from Sigma. DL-P5C was made from a commercially available 2,4 dinitrophenylhydrazine derivative of P5C as described [34] with an additional purification step on an ion exchange column [35]. [U-^{14}C]P5C, prepared enzymatically from [U-^{14}C]ornithine (NEN), was a gift of J. Phang. Restriction endonucleases and other enzymes for molecular biology were obtained from Boehringer Mannheim and used according to manufacturer's specifications.

RESULTS

To clone human P5C reductase cDNAs by complementation, we required the appropriate mutant strain of *S cerevisiae* and a human cDNA library. The yeast strain, DT1100, with a partial deletion of its P5C reductase gene (*pro3*) is auxotrophic for proline. As the source of the transforming DNA, we used HepG2 cell cDNA library, size selected for cDNAs >500 bp, in a yeast expression vector [13]. Because HepG2 cells have high P5C reductase activity (TABLE II), we expected that they would be an adequate source for the reductase cDNA. Transformants were selected on a uracil-free minimal medium supplemented with proline. Approximately 10^5 of these were replica plated onto a minimal medium to screen for proline prototrophs. Two colonies, able to grow in the absence of proline, were identified.

We analyzed these two colonies to determine if the Pro+ phenotype was conferred by sequences on the plasmid. To test for cosegregation of the Ura+ and Pro+ phenotypes, we grew the transformants in a medium permissive for plasmid loss (minimal plus uracil and proline). All Ura- colonies were also Pro-, indicating that the sequence complementing proline auxotrophy was located on the plasmid. To characterize the plasmid inserts, we shuttled the recombinant plasmids from the yeast transformants into *E coli*. Both recombinant plasmids (pPRO+.1 and pPRO+.2) had 1.8 kb inserts that hybridized to one another (data not shown). Reintroduction of pPRO+.1 and pPRO+.2 into DT1100 conferred proline prototrophy, confirming that these plasmids carried *pro3*-complementing activity (FIGURE 1). We designated the pPRO+.1 transformed DT1100 strain as KD100.

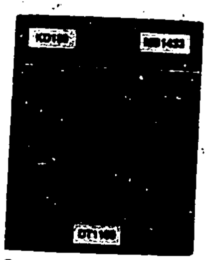

Figure 1. Growth of *S cerevisiae* strains MB1433, DT1100, and KD100 on a proline-free medium supplemented with uracil and tryptophan for four days at 30° C. The position of the strains is indicated.

To be certain that the complementing activity in KD100 was due to P5C reductase, we measured the activity of the enzyme in extracts of DT1100 and KD100. Extracts of DT1100 had no detectable P5C reductase activity. Extracts of KD100 had low but measurable P5C reductase activity (TABLE II) which was approximately 10% that of normal human fibroblasts and 1-3% that of the proline-prototrophic parental strain (MB1433). To confirm that the relatively small amount of product measured in these assays was produced by an enzyme catalyzed reaction, we showed that it increased linearly over time and coeluted from an ion exchange chromatography column with bona fide radioactive proline (data not shown). We conclude that there is P5C reductase activity in the KD100 yeast albeit at levels much lower than those of MB1433. To determine if this low level of P5C reductase activity was sufficient for normal growth, we compared the growth rates of MB1433 and KD100 in minimal medium. The doubling time for both strains was 2.25 h. The DT1100 strain showed no growth under these conditions.

TABLE II: P5C REDUCTASE ACTIVITY IN *S CEREVISIAE* STRAINS AND HUMAN CELL LINES

S cerevisiae strain	Specific Activity* (nmol proline/h/mg protein)
MB14433 (*PRO3*)	3010 (2251 - 3548)
DT1100 (*pro3Δ*)	not detectable
KD100 (*pro3Δ* + pPRO+.1)	63 (22 - 109)
Human cell lines	
fibroblasts - control 1	1218 (1187 - 1241)
fibroblasts - control 2	515 (494 - 533)
HepG2	5143 (4929 - 5357)

* mean and (range) of duplicate determinations for each sample. For the yeast samples, two different preparations were assayed.

The inserts from pPRO+.1 and pPRO+.2 were subcloned into pGEM4, resulting in recombinant plasmids designated phuP5CR.1 and phuP5CR.2. The phuP5CR.1 insert was sequenced in its entirety in both directions (FIGURE 2). The nucleotide sequence of the insert and the derived amino acid sequence are shown in FIGURE 3. We sequenced the termini of the phuP5CR.2 insert. The 3' end was identical to that of phuP5CR.1 while the 5' end had five additional base pairs (FIGURE 3), indicating that pPRO+.1 and pPRO+.2 were two independent isolates. The 1805 bp huP5CR.1 cDNA has an open reading frame of 957 bp if the first AUG is the translational start codon. Use of this AUG preserves regions of amino acid identity with the P5C reductases from other species (FIGURE 4). The 957 bp open reading frame encodes a protein of 319 amino acids with a predicted molecular mass of 33.4 kD. The predicted human reductase amino acid sequence is 32% identical to *S cerevisiae*, 38% to *E coli* and *P aeruginosa* and 44% to soybean.

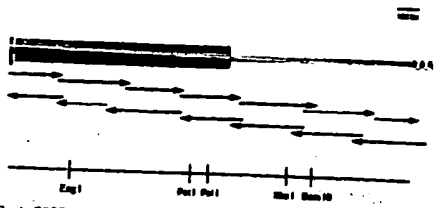

Figure 2. The huP5CR.1 cDNA. The open rectangle denotes the 957 bp open reading frame. The arrows below indicate the sequencing strategy. A simple restriction map is also given. There are no sites for the enzymes Eco RI, Hind III and Rsa I.

FIGURE 5a shows Northern blot analysis of RNA from HepG2 cells, human fibroblasts and various yeast strains probed with huP5CR.1 cDNA. In all human cell line RNA samples, the major hybridizing transcript is 1.65 kb, correlating well with the size of the phuP5CR inserts. Similar results were obtained with RNA from other human cell lines including HeLa, HEK, AD12 and MCF7 (data not shown). The total cellular RNA from both HepG2 cells and fibroblasts have additional minor hybridizing transcripts of 2.25 kb and 1.0 kb. The latter is not present in HepG2 poly A+ RNA. The major hybridizing mRNA in KD100 is 2.0 kb. The slightly larger size of this transcript as compared to that in human cells is due to incorporation of vector glyceraldehyde-3-phosphate dehydrogenase sequences. The intensity of the major hybridizing band in KD100 is ~ 15-fold that in HepG2 cells and ~ 30-fold that in human fibroblasts. As expected, there was no detectable hybridizing transcript in MB1433 or DT1100 despite the presence of approximately similar quantity and quality of RNA in each lane as shown by probing the blot with yeast actin (FIGURE 5b).

Comparison of the amounts of P5C reductase mRNA (FIGURE 5) and enzymatic activity (TABLE II) measured in KD100 extracts shows that the low level of reductase activity in this strain is not due to a low level of the reductase transcript. Rather the low enzymatic activity must be due either to inefficient translation or to post-translational causes. The differences in P5C reductase activity between HepG2 cells and fibroblasts (5 to 10-fold) is partially accounted for by the greater levels of P5C reductase mRNA in HepG2 cells (2 to 3-fold).

Figure 5. An RNA blot of samples from various human and yeast cells. Panel A: Total cellular RNA from HepG2 cells (10 µg) or cultured fibroblasts (10 µg) or the indicated strains of *S cerevisiae* (20 µg) or HepG2 poly A+ RNA (1 µg) was loaded in the indicated lanes. The blot was hybridized with radiolabelled huP5CR.1 cDNA and the autoradiogram exposed for 48 hours. A one-tenth exposure of the KD100 lane is shown on the right. Panel B: The same blot probed with mouse β-tubulin (human lanes) or *S cerevisiae* actin (yeast lanes). Standard size markers (kb) are noted.

We analyzed endonuclease digested human genomic DNA to determine the complexity of the fragments detected with the P5C reductase cDNA probe. 48. One of two major hybridizing fragments were present in digests with five different restriction endonucleases, suggesting a single P5C reductase gene with a relatively simple organization. Hybridizing this blot under less stringent conditions (see METHODS) did not reveal any new fragments (data not shown). Using two human/rodent hybrid cell mapping panels, we localized the P5C reductase gene to human chromosome 17 (data not shown).

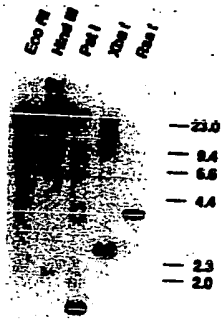

Figure 6. A Southern blot of human genomic DNA hybridized with radiolabelled huP5CR.1. Human genomic DNA (10 µg) was digested with the indicated restriction enzymes. Standard size markers (kb) are noted.